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Photodynamic Effects of Toluidine Blue on Human Oral Keratinocytes and Fibroblasts and *Streptococcus sanguis* Evaluated In Vitro

Nikolaos S. Soukos, Dr Odont, Michael Wilson, PhD, Tracy Burns, BSc, and Paul M. Speight, PhD

Department of Oral Pathology (N.S.S., P.M.S.) and Department of Microbiology (M.W., T.B.), Eastman Dental Institute, London WC1X 8LD, United Kingdom

Background and Objective: **Some oral bacteria are susceptible to killing by red light after their sensitization with toluidine blue O (TBO). The photochemotherapy of periodontal disease in vivo would require a therapeutic window where bacteria could be killed without adjacent normal tissue damage.**
Study Design/Materials and Methods: **The laser-induced effects of TBO on normal human gingival keratinocytes and fibroblasts have been studied in vitro. For the assessment of viability, the CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay was used.**
Results: **TBO was cytotoxic at low concentrations (5.0 μg/ml). Sensitization of keratinocytes and fibroblasts with 2 and 5.0 μg/ml TBO, respectively, for 5 min and exposure to light from a 7.3 mW Helium/Neon (HeNe) laser for up to 2 min (0.876J) did not reduce cell viability. However, killing of *Streptococcus sanguis* was achieved following exposure to HeNe light for 75 sec (0.547J) in the presence of TBO at a concentration of 2.5 μg/ml.**
Conclusion: **The development of a system for the lethal photosensitization of bacteria responsible for periodontal disease may be possible.** © 1996 Wiley-Liss, Inc.

Key words: bacteria, lasers, lethal photosensitization, periodontal disease

INTRODUCTION

The basis of photodynamic therapy (PDT) is the activation of a photosensitizing drug by light, which results in cytocidal effects both in vivo and in vitro [1,2]. PDT has been used for the treatment of tumours, but bacteria also can be killed by light after treatment with an appropriate photosensitizer [3–5]. Recent studies have shown that a number of oral bacteria, including the periodontopathogens *Porphyromonas gingivalis, Eikenella corrodens, Actinobacillus actinomycetemcomitans,* and *Fusobacterium nucleatum* and the cariogenic *Streptococcus mutans, Streptococcus sobrinus, Lactobacillus casei,* and *Actinomyces viscosus,* are susceptible to killing by red light after sensitization with dyes such as toluidine blue O and methylene blue [6–10]. It has also been demonstrated that lethal photosensitization of periodontopathogenic species is possible when in the form of biofilms and when they are present in subgingival plaque samples from patients with chronic periodontitis [11,12]. This implies that the use of low-power lasers in conjunction with an appropriate photosensitizer may be a useful alternative to antibiotics and antiseptics as an adjunct to mechanical methods of supragingival and subgingival plaque control in vivo. Prior to any clin-

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Address reprint requests to Dr. Paul Speight, Department of Oral Pathology, Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK.

ical trial, however, it is important to determine whether host tissues would be affected by light doses and sensitizer concentrations that are effective against bacteria.

The purpose of this study was to investigate the effects of laser light on normal oral epithelial cells and fibroblasts in the presence of toluidine blue O (TBO) in vitro. This was an attempt to find a therapeutic window whereby bacteria could be killed without damaging adjacent normal tissue. *Streptococcus sanguis* was used as a test organism as this is one of the most common species found in dental plaque.

MATERIALS AND METHODS

Cells and Culture Conditions

Normal human gingival keratinocytes and fibroblasts were prepared from gingival biopsies obtained during minor oral surgical procedures.

Gingival keratinocytes were grown on tissue culture plastic in a nutrient medium composed of a 1 + 3 mixture of Ham's F12 (Sigma Chemical Co., Poole, UK) and DMEM (Gibco, UK) supplemented with 1.8×10^{-4}M adenine and 2 mM glutamine (both from Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (both from Gibco), 10% fetal calf serum (Globeharm, UK), 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml EGF, 10^{-10}M cholera toxin, 5 μg/ml transferrin, and 2×10^{-11}M liothyronine (all from Sigma) and 2.5 μg/ml fungizone (Gibco) in the presence of 3T3 feeder previously treated with 10 μg/ml Mitomycin C for at least 2 hr at 37°C in the dark. Medium was changed 2–3 times a week, and keratinocytes were passaged when the average colony size reached ~1,000 cells. Subcultures were made after removing 3T3 feeder cells by exposure to 0.02% EDTA (Sigma) for 5 min and pipetting vigorously. The keratinocyte colonies, which remained adherent, were then disaggregated to single cells using trypsin/EDTA (Sigma) and replated with fresh-treated 3T3 feeder cells.

Gingival fibroblasts were grown in nutrient medium composed of DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone. Medium was changed two times a week. Cells were passaged once weekly using trypsin/EDTA, and 3T3 cells were grown and subcultured in the same way.

All cells were maintained in 75 cm^2 tissue culture flasks with 12 ml medium and kept at 37°C in a humidified 5% CO_2 incubator.

Preparation of TBO

TBO (C.I. 52040) was obtained from Sigma and dissolved in growth medium to give a stock solution at a concentration of 100 μg/ml. It was kept in the dark and further diluted in growth medium prior to use.

Cytotoxic Effects of TBO

Establishment of cultures. Cells were trypsinized in the exponential growth phase and counted using a hemocytometer. Keratinocytes (5,000) or fibroblasts (15,000) were seeded into wells of 96-well culture plates (Sterilin, UK) with 10% fetal calf serum (FCS). These were incubated overnight to allow cells to attach and resume exponential growth, after which time TBO was added. Medium was removed and replaced with growth medium with 2% FCS containing various concentrations of TBO (0.5–50.0 μg/ml) in triplicate. Control cultures received only medium without TBO.

Estimation of cell growth. At 5 min after addition of TBO, medium was aspirated from all the wells and the cells washed with sterile phosphate-buffered saline (PBS) to remove excess TBO, which could cause precipitation later in the procedure. Cell viability was assayed by the reduction of the tetrazolium compound MTS, a method of assessing viable cell numbers [13].

CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay (MTS assay). This assay (Promega, UK) is a colorimetric method for determining the number of viable cells, based on the cellular conversion of the tetrazolium salt MTS into a formazan, in the presence of phenazine methosulphate (PMS). This is soluble in cell culture medium and can be measured on an ELISA plate reader directly from 96-well assay plates without additional processing. Absorbance is directly proportional to the number of living cells in the culture.

Reagents for the CellTiter 96AQ assay were prepared just prior to addition to the plates as follows: 2.0 mls MTS solution and 100 μl PMS solution were combined in a test tube for assessing viability in one 96-well microtiter plate. Growth medium (100 μl with 10% FCS) was added to each well followed by 20 μl MTS/PMS solution. The 96-well plate was covered with aluminium foil and incubated at 37°C in 5% CO_2 for 4 hr. After this time the absorbance of each well was measured using an ELISA reader at 492 nm.

TBO Uptake Determination

Keratinocytes (5,000) and fibroblasts (15,000) were placed in wells of 96-well culture plates and incubated overnight, after which TBO was added to three wells of each to give a final concentration of 2.0 μg/ml (keratinocytes) or 5.0 μg/ml (fibroblasts). After 5 min, the supernatant was aspirated from each well and the cells were gently washed twice with PBS to rinse out excess dye. The dye bound by living cells was extracted by adding 100 μl of 20% ethanol with 0.1 M HCL [14]. The optical density (OD) of each well was read at a wavelength of 570 nm. The % uptake of TBO by cells in relation to the total dye in the growth medium was then calculated.

Exposure to Laser Light

Cells (5×10^3 keratinocytes and 15×10^3 fibroblasts) in 0.1 ml growth medium were pipetted into the wells of 96-well microtiter plates. Cells were allowed to attach for 24 hr, after which the medium was removed and triplicate cell cultures were refed with medium containing TBO (2.0 μg/ml for keratinocytes and 5.0 μg/ml for fibroblasts) for 5 min. The medium was then removed from all wells and replaced by PBS. Each well of a triplicate cell culture was exposed to laser light for 1 (438 mJ) or 2 min (876 mJ). The light source used was a 7.3 mW helium neon (HeNe) gas laser (NEC Corp., Japan), which emitted light in a colimated beam, diameter 1.3 mm with a wavelength of 632.8 nm. After exposure, the cells and controls (cells exposed to light in the absence of TBO, cells treated with TBO but not exposed to light, and cells not exposed to TBO or light) were refed with medium, and growth was determined using the MTS assay as described above.

Preparation of Bacteria

The organism used in this study was *S. sanguis* NCTC 10904. For experimental purposes, cultures were grown anaerobically for 16 hr in tryptone soya broth (TSB) at 37°C.

Lethal Photosensitization of *S. sanguis*

Solutions of TBO in TSB were added to 16 hr cultures of *S. sanguis* to give final concentrations of 5 and 2.5 μg/ml. Controls received only TSB. These were incubated at room temperature for 5 min. A total of 1.0 ml of each bacterial suspension was poured over a tryptone soya agar (TSA) plate, the excess removed, and the plate dried at 37°C for 1 hr. Different areas of the plate were then exposed in duplicate to the HeNe laser light for periods of 30–120 sec. After anaerobic incubation for 24 hr at 37°C, the plates were examined for growth-free zones, then reincubated for 48 hr, and examined for growth within these zones.

RESULTS

Cytotoxicity of Toluidine Blue

The effects of different concentrations of TBO on the growth of cells measured by the MTS assay are shown in Figure 1. Toluidine blue O was cytotoxic at low concentrations with fibroblasts exhibiting a lower susceptibility. The highest concentration of TBO showing no statistically significant effect on viability was 2.0 μg/ml for keratinocytes (Student's t-test; $P > 0.05$) and 5.0 μg/ml for fibroblasts (Student's t-test; $P > 0.05$). The mean reductions in viability after using the above mentioned concentrations in relation to controls were 12.0% and 8.0%, respectively.

Absorbance of TBO by Cells

The % uptake of TBO by cells in relation to the total dye in the medium 5 min after its addition is shown in Figure 2. The % uptake of TBO by keratinocytes and by fibroblasts was 7.5% and 6.9%, respectively.

Laser Irradiation

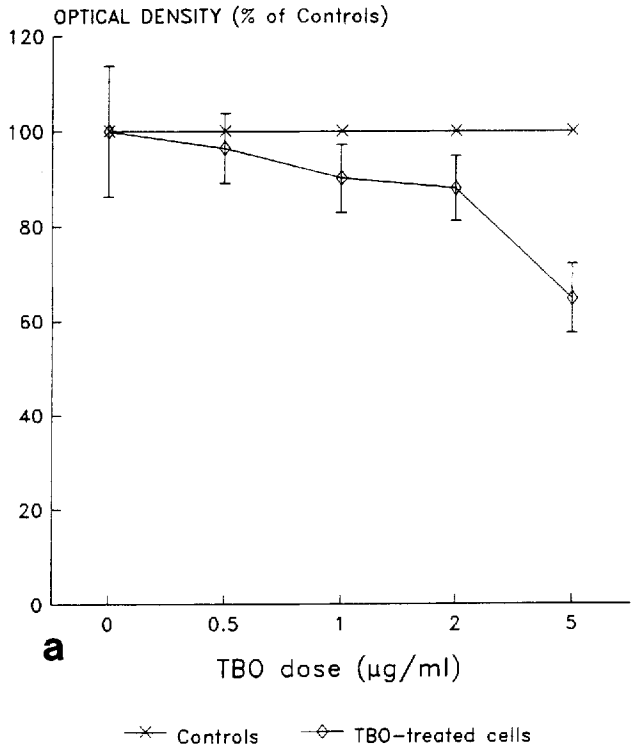
Figure 3 shows the effects of laser light on the viability of gingival keratinocytes and fibroblasts after their sensitization for 5 min with 2.0 μg/ml and 5.0 μg/ml TBO, respectively.

Gingival keratinocytes. Toluidine blue O caused a 5.0% reduction in cell viability in the presence of He/Ne light for 2 min; however, this was not statistically significant (Student's t-test; $P > 0.05$). Laser light alone did not affect cell survival after exposure for 2 min. The viability of nonirradiated TBO-treated cells was reduced by only 4.5%.

Gingival fibroblasts. The reduction in viability of TBO-treated cells after their exposure to laser light for 2 min was 4.5% and was not statistically significant in relation to controls (Student's t-test; $P > 0.05$). There was no reduction in viability after exposure to light alone or to TBO dose in the absence of light.

Lethal Photosensitization of *S. sanguis*

Streptococcus sanguis was killed after exposure to HeNe laser light for 75 sec (energy dose = 547 mJ, energy density = 42.1 J/cm^2) when sen-



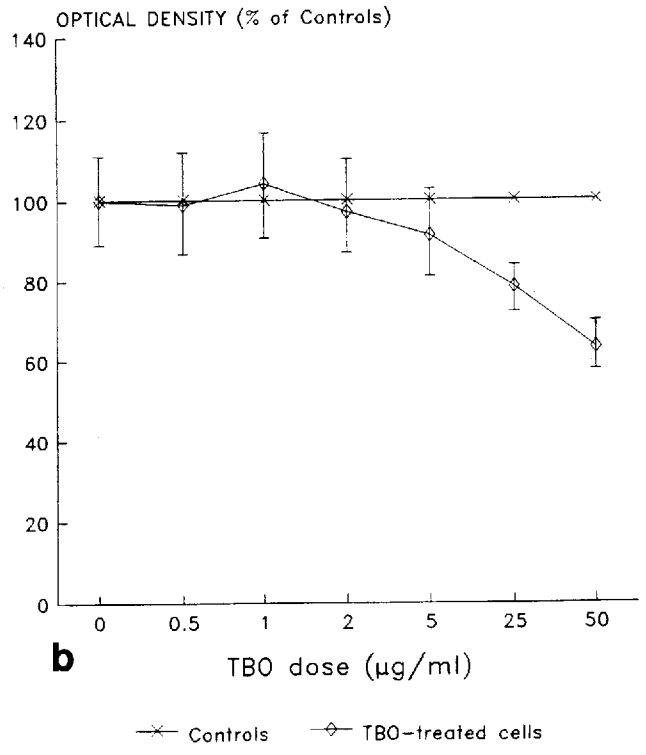


Fig. 1. Cytotoxic effects of different concentrations of TBO on (**a**) gingival keratinocytes and (**b**) fibroblasts as determined by the MTS assay 5 min after the addition of the dye. Values at concentration points represent the means of optical density expressed as % controls (cells untreated with TBO, whose optical density is expressed as 100) and the bars the 95% confidence intervals.

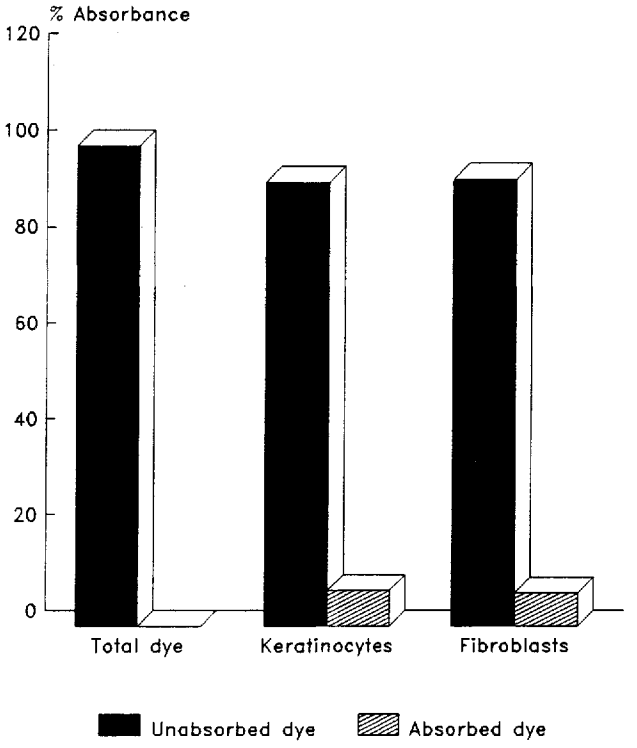


Fig. 2. Uptake of TBO by cells in relation to the total amount of dye added. Values represent means of % uptake.

sitised by TBO at a concentration of 2.5 μg/ml and 5.0 μg/ml (Table 1). Neither the dye nor the laser light alone had any detectable effect on the viability of the organism as there was growth in the presence of the dye on the areas of the plate not exposed to the laser light, and on the control plate exposed to laser light in the absence of the dye.

DISCUSSION

The results of this study have shown that TBO was cytotoxic to human gingival keratinocytes and fibroblasts in vitro and that the cytotoxic effects were dose-dependent. The dye was cytotoxic at quite low concentrations, especially in the case of keratinocytes (concentrations higher than 2.0 μg/ml), fibroblasts showed a lower susceptibility to TBO (5.0 μg/ml). This was somewhat surprising as TBO is used at concentrations 2,000-fold greater than this, without adverse effects, in the diagnosis of premalignant oral lesions in humans [15]. Pilot studies (data not shown) demonstrated that uptake of the dye by cells was extremely rapid. This rapid uptake of TBO and its binding to DNA and RNA [16] may explain these cytotoxic effects. It has also been

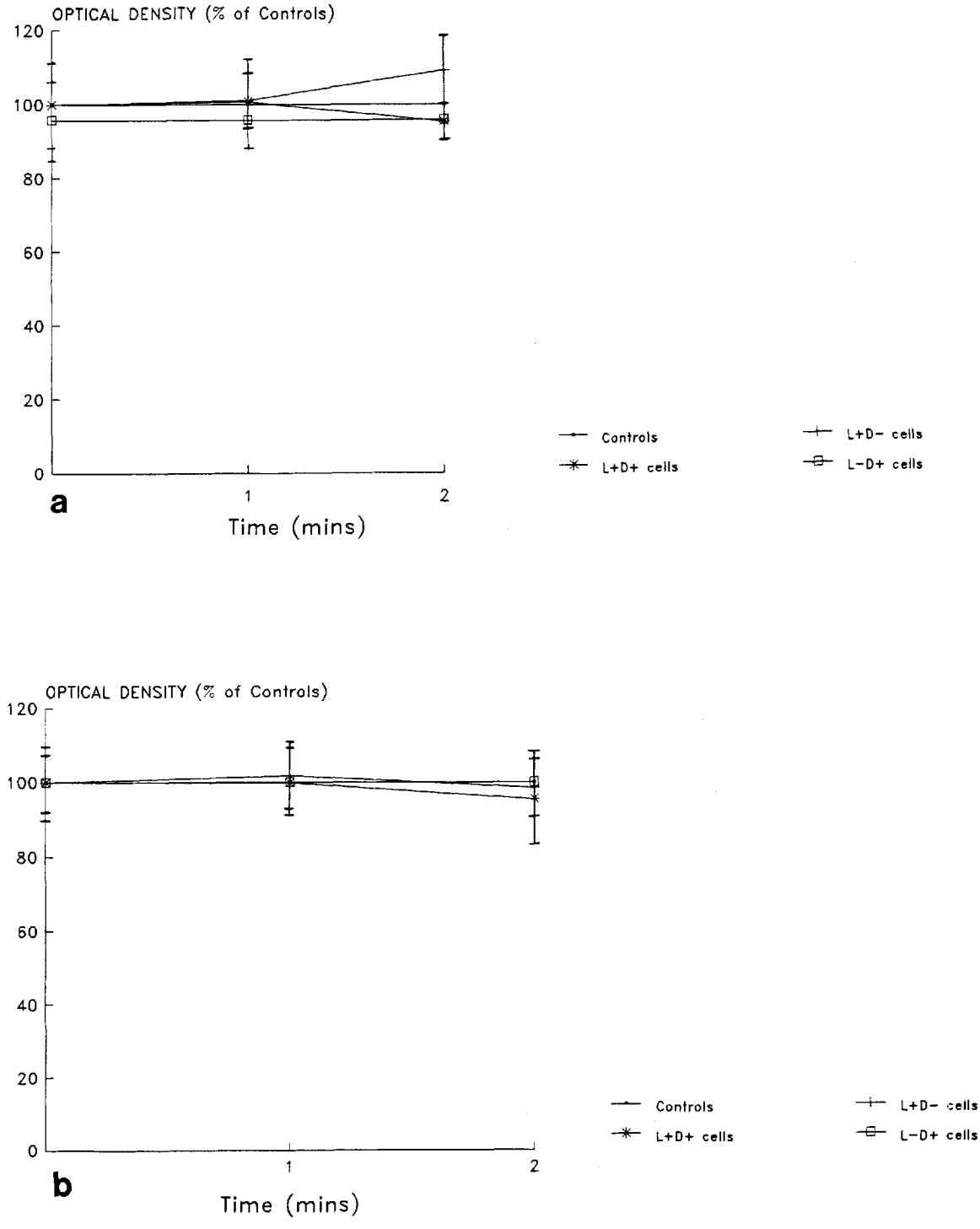


Fig. 3. Growth of TBO-sensitized gingival keratinocytes (**a**) and gingival fibroblasts (**b**) as determined by the MTS assay after their exposure to laser light for various times. Values at time points represent the means of optical density expressed as % of controls (cells untreated with TBO or light, whose optical density is expressed as 100) and the bars the 95% confidence intervals:

L−D− Controls
L+D− TBO-untreated cells but irradiated with laser light
L+D+ TBO-sensitized cells and irradiated with laser light
L−D+ TBO-treated cells but not irradiated with laser light

TABLE 1. Survival of *S. sanguis* Following Exposure to HeNe Laser Light in the Presence of Various Concentrations of Toluidine Blue

	Exposure time (sec)[a]					
TBO (μg/ml)	0	30	60	75	90	120
0	–	–	–	–	–	–
2.5	–	–	–	+	+	+
5.0	–	–	–	+	+	+

[a] + = presence of a growth-free zone; – = absence of a growth-free zone.

reported that the viability of some bacteria decreased with time after exposure to the dye at high concentrations (50 μg/ml) in the absence of laser light [10]. Because of this remarkable cytotoxicity of TBO, a pre-irradiation time of 5 min was chosen prior to exposure to laser light. During this period of time, 7–8% of the dye had been absorbed by the cells without any detectable cytotoxic effects.

The concentrations of TBO that were chosen for lethal photosensitization experiments in the present study were much lower than those found to be effective for killing bacteria in previous studies [6–10]. For many bacteria, the minimum bactericidal concentrations of dye in the absence of laser light were up to 100 μg/ml [10]. It is possible that TBO does not enter the bacterial cells as easily as human cells due to the presence of a cell wall and extracellular structures such as the capsule and slime layers.

Our photosensitization studies clearly showed that after exposure to 2 μg/ml or 5.0 μg/ml TBO, the viability of keratinocytes or fibroblasts, respectively, was not affected by exposure to HeNe light for up to 2 min (0.876 J). However, killing of *S. sanguis* was achieved following exposure to HeNe light for 75 sec (0.547 J) in the presence of TBO at a concentration of 2.5 μg/ml. On the basis of these results, therefore, lethal photosensitization of this major plaque-forming organism would appear to be possible at sensitizer concentrations and light doses that do not affect the viability of keratinocytes or fibroblasts.

It has been reported that TBO can induce a significant increase in chromosome damage [17] and has a mutagenic effect in the in vitro Ames salmonella test [16,18]. Recently, however, it has been demonstrated that toluidine blue has no effect as a carcinogen in the hamster cheek pouch [19]. There are no reports of toxicity to oral rinsing or direct topical use of a 1% toluidine blue solution in humans [15]. An extensive search of the literature showed no other studies concerning the cytotoxicity of TBO to cells in vitro.

Our results are encouraging with regard to the development of an effective approach for the control of plaque-related oral diseases. However, more extensive experiments using TBO and other photosensitizers should be conducted both in vitro and in animal studies to develop a photochemotherapeutic system that would be suitable for clinical evaluation of its effectiveness for the treatment of periodontitis.

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